Modification of Cysteine-Gold Modified Electrode with Hemoglobin I from *Lucina pectinata*: XPS study and Electrochemical Activity for Hydrogen Sulfide

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Abstract
The modification of a gold surface with cysteine (HSC₂H₅NCOOH, Cys) and recombinant hemoglobin I from *Lucina pectinata* (rHbI) was characterized. Self-assembled monolayer (SAM) of cysteine was formed at gold surface using 0.05 mM solution in ethanol. This monolayer was treated with nickel ion (Ni²⁺) from a solution of NiCl₂ 50 mM, and the resulting surface was placed in 0.1 mM hemoglobin I solution to bind the protein. Cyclic Voltammetry (CV) and X-ray photoelectron spectroscopy (XPS) were used to characterize the modified surfaces. The high-resolution XPS from S2p and Cls regions provide further evidence that cysteine is adsorbed to Au. Also, it confirms the presence of nickel ion in the Ni2p region when the monolayer Cys/Au is treated with the solution of NiCl₂. The electrochemical response of rHbI/Ni²⁺/Cys/Au electrode was analyzed by CV technique, showing a pair of well-defined and nearly reversible peaks for rHbI Fe(III)/Fe(II) redox couple at 0.213 V vs. Ag/AgCl. The electrochemical response of rHbI-electrode in presence of hydrogen sulfide (H₂S) was studied, obtaining changes in oxidation and reduction current peaks from the voltammograms. The amperometric response of the rHbI-electrode to H₂S was linear in the range from 90 to 400 nM (n = 7, r² = 0.993). This method provides an alternative procedure of surface modification for immobilization of histidine-tag proteins and future construction of hydrogen sulfide sensors.

Key words: Hemoglobin I; Lucina pectinata; Hydrogen sulfide; Cysteine; Gold electrode.
some hydrogen sulfide in their body; it has been proposed that this gas is used to regulate metabolic activity and body temperature, which would explain the above findings [6]. Furthermore, hydrogen sulfide is a central participant in the sulfur cycle, which is the biogeochemical cycle of sulfur on earth. Sulfur-reducing and sulfate-reducing bacteria derive energy from converting sulfur or sulfate into hydrogen sulfide by oxidizing hydrogen or organic molecules. Other bacteria liberate hydrogen sulfide from sulfur-containing amino acids. Several groups of bacteria can use hydrogen sulfide as fuel, oxidizing it to elemental sulfur or to sulfate by using oxygen or nitrate as oxidant. The purple sulfur bacteria and the green sulfur bacteria use hydrogen sulfide as electron donor in photosynthesis, thereby producing elemental sulfur [7].

Consequently, the detection of hydrogen sulfide has gained significant importance within analytical and biomedical communities as a consequence of its toxicity and the corresponding risk associated. Many techniques such as Chronoamperometry [8,9], UV-vis [10-14], Fluorescence [15-20], Ion Chromatography [18,21], GC-Chromatography [22], Capillary Electrophoresis [23], Inductively Coupled Plasma-Atomic Emission [24], and others not less important, have been commonly used for its analysis. Most of these sensors have relatively good selectivity and fast response. However, it is difficult to get sensors sufficiently stable for exposing them directly to real samples due to decomposition of the electroactive substances, or instability of the formed complexes. Other inconveniences are the poisoning of the electrode surface and the slow removal of modifiers from the surface. Another problem encountered is the commercial unavailability of some modifiers, which limits their extensive application [25]. Currently most H₂S analysis are performed using selective membrane electrodes by polarographic techniques based on the specific electrochemical reaction of HS⁻ ion formed in the first dissociation of H₂S gas dissolved in water with the redox couple Fe(CN)₆³⁻ //Fe(CN)₆⁴⁺. This polarographic hydrogen sulfide sensor, background current resulting from electrolytic conduction of current from anode to cathode occurs as ferricyanide is reduced at the cathode and oxidized at the anode. Because the cathode potential is not as negative as the equilibrium potential of HS⁻, the ferricyanide reduction rate is less than the signal current resulting from sample H₂S [26]. Some of the disadvantages of this system are: the time resolved of the analysis and signal stabilization are extremely large, analyze measurements are achieved indirectly by HS⁻ ion detection, weak and fragile membrane, and the large size equipment required.

On the other hand, it has been widely used short-chain alkanethiols where cysteine is one of the most common linker used for the manufacture of commercial biosensors. Cysteine (Cys) is a non-essential amino acid where the thiol group provides the bond to gold surfaces where the amino and carboxyl moieties are oriented away from the surface of the electrode [27]. Some of the most relevant applications where gold-cysteine surface is involved are the detection of cytochrome c [28], chiral discrimination between 3,4-dihydroxyphenylaniline isomers [29] and studies of electron transfer involving protein-promoter complexes [30]. Also cysteine has a thiol group that is excellent for bonding to gold surfaces, leaving the carboxyl and amino groups exposed to coordinate with metal ions [31,32]. Immuno-proteins have been assembled and immobilized on gold-cysteine surfaces by using these free amino and carboxyl groups [33].

From this previous information, we can deduce that it is possible to immobilize some biomolecules in order to prepare new sensors. This suggests that several proteins can be linked and immobilized to a surface or support throughout the formation of covalent bonds between one or more amino acids of the biomolecule with the surface. We also can attach an intermediate linker that has one extreme bonded tightly to the metal surface and the other extreme of the linker to the protein molecule. Generally, these proteins have groups of high electron density allowing them to establish such links. Recombinant hemoglobin I from *Lucina pectinata* possesses a histidine-tag added by directed mutagenesis techniques, containing imidazole rings of high electron density that are used for purposes of purification. The nickel or cobalt ions present in commercial resins are responsible for the binding process to these proteins. Complexing the nickel ions to the gold-cysteine surfaces allow to bind the hemoglobin by the histidine-tag.

The clam *Lucina pectinata* has three hemoglobin (HbI, HbII, HbIII), where hemoglobin I (HbI) is a monomeric protein composed of 143 amino acids with high hydrogen sulfide affinity [34,35]. Its unique heme pocket contains three residues not commonly found in vertebrate globins:
Phe 29 (B10), Gln 64 (E7), and Phe 68 (E11), which are thought to be important for high affinity for hydrogen sulfide. This hemoglobin is responsible to transport H₂S in the symbiotic cycle present between the clam and bacteria. These bacteria use hydrogen sulfide for their metabolic processes and to protect the clam from H₂S toxicity [36]. Recombinant Hbl (rHbl) is a (His)₆-tagged protein that was cloned and expressed in Escherichia coli. Kinetic studies with Hbl confirmed that rHbl and Hbl have identical binding properties, where the association constant (kₐ) for the clam’s Hbl is 2.73 x 10⁻⁴ M⁻¹ s⁻¹ and for rHbl is 2.43 x 10⁻⁴ M⁻¹ s⁻¹ [37]. This special characteristic of rHbl allows us to postulate it as a protein for the design of a potential biosensor in the quantification of H₂S. This study presents the modification of a gold surface with cysteine, nickel ion (Ni²⁺) and recombinant hemoglobin rHbl using spectroscopic and electrochemical techniques. Hemoglobin rHbl was used because it contains the histidine-tag, required for the bond formation between the nickel ion and the protein, in contrast to Hbl wild type that does not have it. The cyclic voltammetry was used to confirm the modification process, and the electrochemical response of the electrode according to hydrogen sulfide concentration.

2. Materials and Methods
2.1. Materials and Instrumentation
A detailed description of the recombinant Hbl (rHbl), expression and purification procedures has been described in previous works [37]. Recombinant hemoglobin rHbl solutions were prepared by dissolving it in a 0.1 M phosphate buffer (pH 7.50). Additional chemicals (e.g. H₂SO₄, cysteine, ethanol, Na₂S, NiCl₂) were purchased from Sigma–Aldrich and used as received. The water used in our experiments was previously distilled and pumped through a deionization system to give an 18 MΩ cm water. Polycrystalline gold electrodes from Bioanalytical Systems (BASi) Inc., with a 1.6 mm diameter were employed as the working electrodes for the electrochemical experiments. Ag/AgCl electrodes and platinum wire, from BASi, were used as the reference and auxiliary electrodes, respectively. The aqueous solutions were degassed with nitrogen for at least 20 min prior to an electrochemical experiment. The measurements were performed at room temperature on a BASi Epsilon for an Electrochemistry Potentiostat/ Galvanostat system with EC software, connected to a cell stand C-3 for voltammetry. The cell is equipped with a platinum wire auxiliary electrode (7.5 cm) with gold-plated connector (MW-1032), and a 7.5 cm long RE-5B Ag/AgCl reference electrode with a Vycor frit (the filling solution is aqueous 3M NaCl that has been saturated with AgCl). Software: EC epsilon version 1.60.70. The sample XPS analyses were performed using the Al Kα (15.0 kV at 350 watts) source of a PHI 5600 ci spectrometer. This instrument has a hemispherical analyzer, a toroidal monochromator, and a multichannel detector. The base pressure in the chamber during analysis was less than 1x10⁻⁹ Torr. The binding energy values reported in this study were corrected using the C 1s signal of the atmospheric contaminants (284.5 eV). Different experiment setups were used for the XPS studies. The conditions for each setup are summarized in the results analysis section.

2.2. Gold surfaces pre-treatment
All surfaces used were new and before their modification they were submitted to a cleaning treatment. MAXTEK® Au surfaces were used for XPS experiments. These crystals are composed of 2000 Å of gold on top of a polished AT-cut quartz crystal, using a thin film of chromium or titanium as an under layer to improve adhesion between gold and the quartz crystal. Commercial polycrystalline gold disk electrodes (Bio-Analytical System, BASi) of 1.6 mm of diameter were used to carry out the electrochemical measurements. The polycrystalline gold electrodes used for cyclic voltammetry and surfaces for XPS measurements, were prepared by polishing the surfaces with 1.0, 0.3, and 0.05 mm Al₂O₃ paste, and then rinsed copiously with deionized water. We verified its cleanliness by cycling between 0.2 and 1.5 V in 0.1 M H₂SO₄ at 100 mV/s, until reproducible voltammograms were obtained [38]. Following the pre-treatment procedures, these electrodes were cleaned with deionized water and any excess of water was removed with nitrogen gas.

2.3. Electrode modification
Self-assembled monolayers of cysteine were prepared by immersing a clean gold surface in a recipient containing 0.5 mL of 50 mM cysteine in anhydrous ethanol for 24 h. Then the surface was rinsed with deionized water and immersed immediately into 50 mM NiCl₂ aqueous solution for 2 h. After that, the surface was rinsed with deionized water and immersed into 100 mM rHbl aqueous solution for 72 h to obtain the hemoglobin modified gold surface, which was stored in phosphate buffer pH 7.50 at 4°C until further use.

2.4. Electrochemical measurements
Cyclic voltammetry technique was used to identify changes in current–potential shifts throughout the modification process. Also current changes were evaluated in the modified hemoglobin-electrode when aliquots of a Na₂S solution were added in order to provide H₂S. In all cases a 0.1 M phosphate buffer solution pH 6.70 was used, and the voltammograms were obtained at 100 mVs⁻¹. The amperometric analysis was carried out by applying potential of 0.3 V vs. Ag/AgCl on a stirred cell at 25°C. The response was measured as the difference between total and residual currents. The sodium sulfide solution was prepared by adding the salt, previously purged with N₂, to degassed and deoxygenated water to prevent oxygen contamination.

3. Results and Discussion
3.1. XPS characterization
It is extremely important to establish that the cysteine monolayer has formed and that most of the molecules are chemisorbed to the gold surface. For this purpose, apart from cyclic voltammetry, XPS technique was used for the characterization. X-ray photoelectron spectroscopy (XPS) is a technique that has been widely used to study the composition of self-assembled monolayers on metallic surfaces. This technique was used to characterize the monolayer Cys/Au when the gold surface was exposed to a cysteine solution in anhydrous ethanol for 24 hours. Similarly, this technique was used to establish the presence of Ni²⁺ ion and formation of the surface Ni²⁺/Cys/Au when the monolayer was placed in a solution of NiCl₂ to form the
complex between cysteine and Ni²⁺ ions. The XPS binding energy spectra of the (a) clean gold and (b) treated with 50 M cysteine in ethanol are shown in Fig. 1. The spectrum in Fig. 1b presents the binding energy peaks that were attributed to Au4f, C1s, O1s, S2p, which are present in the gold surfaces treated with the Cys solution. The XPS spectrum in Fig. 1a, has the same XPS signals as that presented for the Cys/Au modified surface, with the exception of the S signal. The S binding energy peaks confirm the modification of the gold surface with Cys. This is typically observed when the compound is adsorbed at the Au surface through the thiol group. Figure 1a shows that the C1s signal corresponding to the clean gold is present, and this is possibly due to the contamination of the environment with organic compounds, which can have the same or higher number of carbon atoms compared with cysteine [39]. Also, Fig. 2(I) shows a HRXPS comparison between (a) Au and (b) Cys/Au in the S2p region, denoting the difference in signal for both spectra due to appearance of a broad peaks centered approximately at 163 eV in Cys/Au spectrum, corresponding to one doublet (S2p3/2 and S2p1/2) with a peak separation of 1.2 eV and area ratio 2:1. These peaks may deconvolution while maintaining the above characteristics in terms of splitting and area ratio, using Gaussian curves. This doublet consists of two components, S1 and S2, where the main component S1 occurs at 161.2 eV while the minor one, S2, is found at 163.3 eV (Fig. 2(II)). The main component S1 is attributed to Cys molecules chemisorbed on gold and indicates the formation of a thiolate species. This component is characteristic of organosulfur compounds on gold reported for cysteine [40,41] and for thiols with unsubstituted alkyl chains and with chains containing aromatic moieties. The second component S2, located near 164.0 eV could be assigned to Cys molecules not bound to gold, that is, physisorbed molecules forming a partially occupied upper layer [42]. In addition, Fig. 2(III) shows a HRXPS comparison for (a) Au and (b) Cys/Au surfaces in C1s region. In Cys/Au spectrums, it is observed the formation of several overlapping peaks that are not present in clean gold. Fig. 2(IV) shows the deconvolution to the peaks present in the signal in the region C1s, and it is evidence the presence of characteristic peaks for the -CH₂ groups (284.8 eV), C-S (286.3 eV) and C=O (288.4 eV) [40,41]. This definitely confirms the adsorption of the cysteine on the gold surface. Furthermore, to confirm clearly the presence of Cys on the gold surface in Cys/Au assembly, a high resolution spectra in Au4f and O1s regions were performed. Fig. 3(A) shows the comparison between the spectra in the region Au4f for (a) Cys/Au surface and (b) clean gold. The spectrum corresponding to Cys/Au surface (a) shows a well-defined doublet with a peak separation of 3.6 eV and a half-width of 1.6 eV. The peak Au4f7/2 shows a binding energy of 84.0 eV which is the typical value expected when thiolate form self-assembled monolayers on Au surfaces. Furthermore, decrease on the peak height and area is an indication that these compounds are adsorbed on
the gold surface blocking the XPS detection of Au4f photoelectrons. The high resolution XPS spectrum for O1s region in Fig. 3(B) shows a slightly asymmetric broad peak centered about at 532 eV for Cys/Au surface. This peak can be formed by the contribution of the oxygen of the carboxyl and -OH groups, previously reported in the literature [44]. In addition, this slight asymmetry may be due to the presence of water molecules coadsorbed on the surfaces. The oxygen (O1s) and carbon (C1s) signals, which appear on the clean gold surface, correspond to contaminants from the air, from the cleaning process, or from handling of the substrate [39]. Moreover, the surface modification obtained between Cys/Au surface with Ni2+ ions was followed by XPS. Fig. 4 presents the curve-fitted HRXPS spectrum for the binding energy region of Ni2p in the modified surface Ni2+/Cys/Au. The Ni2p peaks appear at binding energies of 856 eV (Ni–O) and 861 eV (satellite peak) consistent with previously reported in the literature [45], indicating the presence of a Ni–O bond on the modified Cys/Au surface. For hemoglobin-modified surface rHbI/Ni2+/Cys/Au, was not possible to detect either the presence of Ni2+ ions from a Ni2+-N (histidines) bond or the presence of iron from the porphyrin under these analysis conditions. Eventually, the body volume of the hemoglobin may interfere with the penetration of X-rays, acting as a multilayer composite where bonds cannot be observed in lower layers. This surface was analyzed with different take-off angles and the results were similar.

3.2. Cyclic Voltammetry (CV) studies

Figure 5 shows the differences between the voltammograms for gold electrode modified with cysteine (dashed line), nickel ion (dotted line) and hemoglobin I (solid line) in phosphate buffer solution pH 6.70. In Cys/Au assembly a progressive oxidation current increase from 400 to 600 V vs. Ag/AgCl is observed when compared with the gold bare, suggesting the presence of cysteine on the metal surface. Previous work has already recognized and characterized this kind of surfaces [46, 47]. When this surface interacts with the nickel ions is observed broad oxidation and reduction peaks, which possibly can be attributed to the strong interaction between the carboxyl groups with nickel ions. It is well established that the complex formation concerning proteins histidine-tagged with resins containing nickel or cobalt ions can be used for purposes of purification [48,49]. Histidines exhibit highly selective coordination with nickel and cobalt ions and under physiological pH conditions, binds by non-bonding electron pairs of the imidazole nitrogens with the half empty orbitals of these ions. Originally the modification of Ni2+/Cys/Au with rHbI was more convenient to be done at weakly basic pH (at 7.50) to favor the formation of a covalent bond between the imidazoles of histidine-tag of recombinant hemoglobin and nickel ion-cysteine complexes. Then, this structure was exposed to recombinant hemoglobin for 72 h, producing a drastic change in the voltammogram (Fig. 5, solid line) showing a redox couple. The half wave potential (E1/2), calculated from the average value of the anodic (Ean = 0.177 V vs. Ag/AgCl) and cathodic peak (Ecath = 0.250 V vs. Ag/AgCl) potentials, is 0.213 V vs. Ag/AgCl (E1/2 = 0.004 V vs. NHE). In the literature there is diverse data about different redox potentials of heme proteins when used in different media. As reference hemeprotein we used myoglobin, because they are structurally similar. Table 1 summarizes some of the redox potential found for myoglobin in different media surfaces.

Table 1. Redox potential of myoglobin immobilized on some supports or surfaces.

<table>
<thead>
<tr>
<th>Myoglobin supports</th>
<th>E° (V) vs. NHE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanoporous ZnO [50]</td>
<td>−0.053</td>
</tr>
<tr>
<td>Myoglobin-Au-ITO [51]</td>
<td>−0.007</td>
</tr>
<tr>
<td>Colloidal gold nanoparticles [52]</td>
<td>−0.131</td>
</tr>
<tr>
<td>Ni/NiO [53]</td>
<td>0.0895</td>
</tr>
<tr>
<td>Sol-gel [54]</td>
<td>−0.099</td>
</tr>
</tbody>
</table>
sequence, and both proteins in the metaquo form (ferric state) have similar spectral properties. Although these proteins are structurally very similar, they differ in the internal amino acids composition around the heme group.

On the other hand, this rHbl-electrode achieved good response when the sodium sulfide solution is added. Fig. 6 shows the cyclic voltammetric behavior of the rHbl/Ni²⁺/Cys/Au electrode observing a decrease of anodic and cathodic currents. Previous works have shown that rHbl has the ability to bind hydrogen sulfide obtained from a sodium sulfide solution. When Na₂S is added to a buffer solution pH<6.9 buffer solution, hydrogen sulfide is released [55]. The H₂S concentrations were calculated based on the H₂S dissolved in the solution, which is only one component of the total sulfide equilibrium. The equation involved in the calculation corresponding to the total sulfide equilibrium system is 

\[ [H₂S] = [Na₂S] / (1 + (K₁[H⁺]) + (K₂[H₂S])) \]

where \( pK₁ = 6.89 \) and \( pK₂ = 19 \) [1]. In our case, the equilibrium of \( H₂S \) system from sodium sulfide is shifted toward the formation of \( H₂S \) because the pH of solution is less than \( pK₁ \).

The decreasing in anodic and cathodic currents during consecutives voltammetric cycles as consequences of the addition of sodium sulfide is an indication of the interaction of the hemoglobin molecules on the modified surface with the hydrogen sulfide produced in solution. As the concentration of sodium sulfide increases with the successive addition to the buffer solution, the voltammograms reaches constant waves. This behavior suggests that the heme pocket for hemoglobin molecules are binding hydrogen sulfide molecules, reaching saturation. It is well known that hemoglobin molecules are associated with hydrogen sulfide to form the complex rHbl-H₂S [55]:

\[ rHblFe(III) + H₂S \rightleftharpoons rHblFe(III–)H₂S \]

Likewise, this behavior indicated that the rHbl kept its native structure capable to bind \( H₂S \) after being immobilized onto the electrode. Due to the fact that there is a change in the amplitude of the signal but there is no change in the potential range of the wave, we conclude that the oxidation number of hemoglobin remains constant. The form in which hemoglobin I is associated to \( H₂S \) has been studied previously [35,36,55-57], where it has been shown that the porphyrin preserves its oxidation state (rHbl metaquo, Fe³⁺) when it interacts with \( H₂S \). When an \( H₂S \) molecule binds to the hemoglobin-Fe³⁺, forming the species rHblFe(III)–H₂S, it apparently prevents it from performing the redox process rHblFe³⁺/rHblFe²⁺, because the link established between the ferric hemoglobin and hydrogen sulfide is stable. Therefore, this entails a decrease in hemoglobin concentration on the surface every time an \( H₂S \) molecule enters, this behavior being observed in the reduction of voltammetric wave currents. Furthermore, the amperometric response of rHbl/Ni²⁺/Cys/Au with successive additions of Na₂S to 0.1M phosphate buffer solution pH 6.70 is shown in Fig. 7. The linear response range of \( H₂S \) concentration was from 90 nM to 400 nM with a correlation coefficient of 0.993 (n = 7). From the slope of -0.0081 nA/nM, the lower limit of detection was estimated to be 27 nM at a signal to noise ratio of 3, and the
lower limit of quantification of 89 nM. This electrode can be reused when is placed in a phosphate buffer solution pH 6.70 at 0.00 V vs. Ag/AgCl for 1 h or until the current stabilizes. Moreover, this electrode lost only 6% of its initial activity after more than four successive measurements and is stored in 0.1M phosphate buffer solution pH 6.70 at 4 °C, when not in use. Also, their relative response current was examined by checking periodically. The hemoglobin-modified electrode retained 95% of activity within a storage period of 30 days under these conditions, but after a storage period of 90 days the sensor showed loss of activity for H₂S. Also, the rHbl-electrode holds good response to a mixture of ions 1 mM Ca²⁺, Na⁺, K⁺, NH₄⁺, NO₃⁻, SO₄²⁻, Cl⁻, Br⁻, I⁻ and 100 mM PO₄³⁻ (from buffer). It should be emphasized that the higher the concentrations of these ions the more significant will be the loss of electrode activity. It is possible that hemoglobin has been denatured by high salinity. On the other hand, erratic response is observed when to the buffer solution was added a small aliquot of a solution 1 mM CN⁻. Cyanide is a ligand that binds strongly to ferric ion forming a very stable complex with hemoglobin, and cannot be displaced by ligands such as O₂, NO or H₂S. Some publications in hemeproteins functionality and dynamic confirm this fact [58]. On the other hand, when is adding a small aliquot of 1 mM H₂O₂ to a phosphate buffer solution, a similar behavior is observed. Several recent studies have demonstrated that monomeric hemeproteins similar to rHbl, may be associated with ferric ion of the porphyrin [59], but as the peroxide concentration increases, could interact with any part of the assembly structure, observing erratic response of the electrode. In conclusion, for the analysis of H₂S is recommended to remove hydrogen peroxide and cyanide, both of which can greatly interfere with the analysis. The problem is that the iron from the porphyrin in these hemeproteins binds to these ligands in the ferric state, and the oxidation state does not change. The effective nuclear charge of ferric ion may change slightly when it forms bonds with such substrates, but potential changes are not so extreme as to be detected with the conditions employed in this study.

4. Conclusions
This work presents a method for the preparation of a modified gold electrode based on the combination of self-assembly technology and metal affinity for histidine-tagged proteins. The modified hemoglobin I gold electrode showed an electrochemical activity to H₂S without the aid of any mediator. During the fabrication of the modified electrode, rHbl was successfully bound to the surface by nickel ions on cysteine-film-modified gold surface. The resulting system rHbl/Cys/Ni²⁺/Au has a promising potential for the construction of an electrochemical biosensor based on the direct electrochemistry of other histidine-tagged proteins or enzymes.

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